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Citation for published version:

Florens, L, Korfali, N & Schirmer, EC 2008, 'Subcellular fractionation and proteomics of nuclear envelopes', *Methods in Molecular Biology*, vol. 432, pp. 117-37. https://doi.org/10.1007/978-1-59745-028-7_8

Digital Object Identifier (DOI):

[10.1007/978-1-59745-028-7_8](https://doi.org/10.1007/978-1-59745-028-7_8)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Methods in Molecular Biology

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Subcellular Fractionation and Proteomics of Nuclear Envelopes

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Running Head: Subtractive Proteomics of Nuclear Envelopes

Abstract

Because of its many connections to other cell systems, the nuclear envelope is essentially impossible to purify to homogeneity. To circumvent these problems, we developed a subtractive proteomics approach in which the fraction of interest and a fraction known to contaminate the fraction of interest are separately analyzed and proteins identified in both fractions are subtracted from the dataset. This requires that the contaminating fraction can be purified to homogeneity. In this case, microsomal membranes are used to represent endoplasmic reticulum contamination, allowing the identification of transmembrane proteins specific to the nuclear envelope. To circumvent problems commonly associated with analyzing membrane proteins, the Multi-dimensional Protein Identification Technology (MudPIT) proteomics methodology is employed.

Key Words: Multi-dimensional Protein Identification Technology (MudPIT); liquid chromatography; tandem mass spectrometry; nuclear envelope; microsomal membranes; transmembrane proteins; inner nuclear membrane; nuclear lamina.

1. Introduction

The nuclear envelope (NE) is comprised of a double membrane, its integral proteins, and the intermediate filament lamin polymer that underlies the membrane and binds several of its integral proteins. While the inner nuclear membrane is clearly a subdomain of the nucleus, the outer nuclear membrane is both the outermost layer of the nucleus and a subdomain of the endoplasmic reticulum with which it is continuous. As a result, the nuclear envelope is highly complexed with both cytoplasmic membranes and filament systems as well as chromatin (1-3). These many interconnections render biochemical isolation of pure nuclear envelopes effectively impossible. To circumvent this, we developed a subtractive proteomics approach (4). Two fractions are separately analyzed: 1) a nuclear envelope-enriched fraction and 2) a fraction known to contaminate nuclear envelopes, but which can be purified completely clean from nuclei. In this case microsomal membranes (MMs) were chosen as the contaminating fraction because they can be cleanly separated from intact nuclei at an early stage in the preparation (5). To purify nuclear envelopes, nuclei are first isolated taking advantage of their mass and density (6), though many other cytoplasmic membranes and filament systems co-purify. A large percentage of the contaminating membranes are removed by floating them on sucrose cushions that are penetrated by the dense nuclei. Finally, the nucleoplasmic contents are removed by swelling nuclei, digesting chromatin, and washing away released chromatin again by pelleting the larger / heavier nuclear envelopes through sucrose (7, 8). Microsomal membranes are purified by breaking the plasma membrane and vesiculating ER with dounce homogenization, removing nuclei and mitochondria by pelleting, and floating microsomal membranes on dense sucrose (5, 9, 10). Due to the complexity of the

nuclear envelope, subsequent extraction steps may be employed depending on the goals of a particular study; however, it should be noted that all additional extractions carry with them the possible loss of true NE proteins. Some subsequent purification procedures are based on the biochemical properties of the nuclear lamin polymer, which, together with associated transmembrane proteins, remains insoluble in the presence of the relatively high concentrations of salt and detergent used here (for review on lamin properties see (11)). Other procedures rely on the solubility properties of membrane proteins and here, we describe alkali extraction to enrich for transmembrane proteins.

Multi-dimensional Protein Identification Technology (MudPIT) (12-14) was used to circumvent several inherent difficulties of working with membrane proteins. In shotgun proteomics, a complex protein mixture is digested into an even more complex peptide mixture. This step may seem counter-intuitive but in actuality, peptides' physicochemical properties are more homogenous than proteins'. In particular, peptides can readily be separated by simple reverse phase chromatography techniques and their molecular weights are ideal to be analyzed by mass spectrometry. In addition, with the advent of tandem mass spectrometry, amino acid sequences can be deduced from peptide fragmentation patterns using software such as SEQUEST (15). SEQUEST peptide level information is reassembled into protein lists using software such as DTASelect (16), while multiple protein lists are compared using software such as CONTRAST (16). Proteins appearing in both NE and MMs fractions are subtracted from the dataset, leaving an in silico "purified" nuclear envelope-specific dataset ("subtractive proteomics"). This NE-specific list of proteins can then be queried for the presence of transmembrane proteins. All 13 previously known NE integral proteins (NETs) were readily identified, as well as all known

components of the nuclear pore complex. An additional 67 hypothetical transmembrane proteins were identified. All eight of these novel putative NETs originally tested targeted to the NE (4), while subsequent characterizations have thus far confirmed the localization of nearly two dozen more NETs (17, 18, W. E. Powell, V. Lazou, D. Kavanagh, P. Malik, N. Korfali, and E. Schirmer, unpublished results).

2. Materials

2.1. Preparation of Tissue (Rodent Livers)

2.1.1. Animals

1. Volumes in the protocol are given based on grams of liver or OD of nuclei. To estimate how many animals to use: ~5 g of liver can be obtained from one rat and ~1.25 g of liver can be obtained from one mouse. We generally produce 1,000 to 2,000 OD of nuclei (1 OD = 3,000,000 nuclei) from the livers of 10 rats (*see Note 1*).
2. 6-8 week old rats (e.g. Sprague-Dawley or equivalent) or mice (e.g. CB6F1/J or equivalent) (*see Note 2*).

2.1.2. Hardware

1. Guillotine or equivalent local method for euthanizing animals.
2. Dissection scissors, scalpel, and forceps/ tweezers.
3. Two beakers on ice, one with 200 mL of double distilled H₂O and the other with 200 mL of 0.25 M SHKM buffer (*see Note 3*).
4. Appropriate materials for covering surfaces during procedure and for cleaning and waste disposal.

2.1.3. Solutions

1. 0.25 M SHKM: 50 mM HEPES-**KOH**, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 250 mM sucrose, and freshly added 2 mM DTT (dithiothreitol) and 1 mM PMSF (phenylmethylsulfonyl fluoride; from a 100 mM solution in ethanol) (*see Note 4*).

2.2. Preparation of Nuclear Envelopes

2.2.1. Hardware

1. Potter-Elvehjem homogenizer with a motor-driven Teflon pestle providing 0.1 to 0.15 mm clearance and the drive motor capable of 1500 rotations per minute (e.g. Potter S Homogenizer catalogue numbers 853 3032 (motor), 854 2600 (60-mL homogenizer cylinder), and 854 3003 (Plunger made of PFTE) from Sartorius or equivalent).
2. Loose fitting (Wheaton type B pestle) glass dounce homogenizer with clearance of between ~0.1 and 0.15 mm.
3. Swinging-bucket rotor with a tube capacity of at least 200 mL if processing 10 rats (e.g. Beckman Coulter SW28 rotor with Beckman Coulter 344058 Ultra-Clear 25 x 89 mm centrifuge tubes).
4. Local standard light microscope, glass slides, and coverslips.
5. Assorted beakers, 2 funnels, and several spatulas.
6. Sterile cheesecloth (*see Note 5*).
7. Large bore luer lock stainless steel needles (e.g. 14 gauge) of greater length than centrifuge tubes and glass luer lock syringes.

2.2.2. Solutions

Solution names include the initials for the primary components: S for sucrose, H for HEPES-**KOH**, K for KCl, and M for MgCl₂ (*see Note 6*).

1. DNase (e.g. Sigma DNase I D4527) resuspended at 10 U/ μ L in H₂O.

2. RNase (e.g. Sigma RNase A R4875) resuspended in H₂O at 10 mg/ mL and boiled for 20 min.
3. Protease inhibitors (*see Note 7*): all solutions require freshly added 1 mM PMSF, 1 µg/ mL aprotinin (from a 1 mg/ mL stock in H₂O), 1 µM pepstatin A [from a 1 mM stock in DMSO (dimethyl sulfoxide)] and 10 µM leupeptin hemisulfate (from a 10 mM stock in H₂O) (*see Note 8*).
4. 0.25 M SHKM: 50 mM HEPES-**KOH**, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 250 mM sucrose, and freshly added 2 mM DTT (dithiothreitol; from a 1 M solution in H₂O) and protease inhibitors. This is the same solution used to wash the freshly isolated livers, except that additional protease inhibitors are added to the fresh buffer in which homogenization occurs.
5. 2.3 M SHKM: 2.3 M Sucrose, 50 mM HEPES-**KOH**, pH 7.4, 25 mM KCl, 5 mM MgCl₂, and freshly added 2 mM DTT (*see Note 9*).
6. 30% SHKM: 0.9M Sucrose, 50 mM HEPES-**KOH**, pH 7.4, 25 mM KCl, 5 mM MgCl₂, and freshly added 2 mM DTT and protease inhibitors (*see Note 10*).
7. 30% SHM buffer: 0.9 M sucrose, 10 mM HEPES-**KOH**, pH 7.4, 5 mM MgCl₂, and freshly added 2 mM DTT and protease inhibitors.
8. 10% SHM buffer: 0.3 M sucrose, 10 mM HEPES-**KOH**, pH 7.4, 5 mM MgCl₂, and freshly added 2 mM DTT and protease inhibitors.

2.3. Preparation of Microsomal Membranes

2.3.1. Hardware

1. The same hardware is required as for *Preparation of Nuclear Envelopes*.
2. A Ti45 fixed angle rotor or equivalent that can provide 150,000 g and matching tubes.

2.3.2. Solutions

The same sucrose solutions used for *Preparation of Nuclear Envelope* can be used in preparing microsomes. In particular the 2.3 M SHKM, 0.25 M SHKM, and a mixture of the two to 1.9 M sucrose will be required.

2.4. Extraction of Fractions

2.4.1. Hardware

1. TLA100.3 rotor for table-top ultracentrifuge or equivalent and corresponding tubes (e.g. Beckman-Coulter 343778 polycarbonate 11 x 34 mm tubes).

2.4.2. Solutions

1. Salt/ detergent extraction: Octyl β -D-glucopyranoside (also called n-Octyl glucoside; Sigma O9882) resuspended at 1% (w/v) in a solution containing 25 mM HEPES-**KOH**, pH 7.5, 400 mM NaCl.
2. Alkaline extraction: 0.1 N NaOH, 1 mM DTT.

2.5. Determining the Purity/ Quality of Fractions

1. Standard labware for Western blotting and antibodies to lamins, characterized integral nuclear envelope and endoplasmic reticulum proteins.
2. Electron microscope facility and glutaraldehyde for fixation.

2.6. Preparation and Digestion of Proteins for MudPIT

2.6.1. Hardware

1. pH indicator strips, 7.5 to 14 (EMD Chemicals, Part # EM-9587-3; <http://www.emdchemicals.com/>).

2. Eppendorf Thermomixer and Thermomixer block for 1.5-mL tubes (Eppendorf, Part # 5355 000.011; <http://www.eppendorf.com/>).

2.6.2. Solutions

1. Ammonium bicarbonate (Sigma-Aldrich, Part # A6141; <http://www.sigma-aldrich.com>), as a 1 M solution in double distilled H₂O, pH adjusted to 8.5, stored at 4°C.

2. Urea, solid (Sigma-Aldrich, Part # U 1250).

3. 90% formic acid (J.T. Baker, Part # JT0129-1; <http://www.jtbaker.com>).

4. Cyanogen bromide (Sigma-Aldrich, Part # 48,143-2).

5. Ammonium hydroxide solution, NH₄OH (Sigma-Aldrich, Part # A6899, in H₂O at 0.9 g/mL density).

6. Tris(2-Carboxylethyl)-phosphine hydrochloride, TCEP (Pierce, Part # 20490; <http://www.piercenet.com/>), as a 1 M stock in HPLC grade H₂O, stored at -20°C.

7. Iodoacetamide, IAM (Sigma-Aldrich, Part # I 1149), made fresh weekly as a 500 mM stock in double distilled H₂O, and stored at -20 °C.

8. Endoproteinase LysC, sequencing grade (Roche Applied Science, Part # 11047825001; <http://www.roche-applied-science.com/>), as a 1 µg/µL stock in double distilled H₂O, stored at -20°C.

9. Calcium chloride (EMD Chemicals, Part # EM-3000), as a 500 mM stock in double distilled H₂O, stored at room temperature.

10. Poroszyme bulk immobilized trypsin, bulk media (Applied Biosystems, Part # 2-3127-00; <http://www.appliedbiosystems.com/>), stored at -20°C.

2.7. Packing and Loading of Microcapillary Column

2.7.1. Hardware

1. SPEC-PLUS PTC18 cartridges (Varian / Ansys Technologies Inc., Part # 572-03; <http://www.varianinc.com/>)
2. 1-mL syringes (Becton, Dickinson and Co, Part # BD309602; <http://www.bd.com>).
3. SpeedVac concentrator (Thermo Electron, Part # SPD111V; <http://www.thermo.com/>).
4. Micropipette laser-based puller (Sutter Instrument Co, Part # P-2000; <http://www.sutter.com/>).
5. Polyimide-coated fused silica capillary, 100 μm i.d. x 365 μm o.d. (Polymicro Technologies, Part # TSP 100375; <http://www.polymicro.com/>).
6. Column scribe (Chromatography Research Supplies, Part # 205312; <http://www.chromres.com/crs/>).
7. Helium pressure cell (custom-made, MTA for blueprints available by request from John Yates, Scripps Research Institute, La Jolla, CA; or Brechbuehler, Inc., Part # 1 100 110; <http://www.brechbuehler.com/>).

2.7.2. Solutions

1. HPLC grade methanol (EMD Chemicals, Part # EM-MX0488-6).
2. HPLC grade acetonitrile (EMD Chemicals, Part # EM-AX0142-6).
3. Glacial acetic acid (Sigma-Aldrich, Part# A 6283).
4. 90% formic acid (J.T. Baker, Part # JT0129-1).
5. Ammonium acetate (J.T. Baker, Part # JT0599-8).
6. 5- μm Polaris C₁₈A reversed phase column (Varian/Metachem Technologies, Part # 2000-250X046; <http://www.varianinc.com/>) (*see Note 11*).
7. 5- μm Partisphere strong cation exchange column (Whatman, Part # WC4621-1507; <http://www.whatman.com/>) (*see Note 11*).
8. Buffer A: acetonitrile/formic acid/double distilled H₂O, 5/0.1/95, v/v.

2.8. Liquid Chromatography in-line with Tandem Mass Spectrometry

2.8.1. Hardware

1. Agilent 1100 series G1379A degasser, G1311A quaternary pump, and G1323B controller (Agilent Technologies; <http://we.home.agilent.com/>).
2. LCQ DECA tandem mass spectrometer (Thermo Electron).
3. Nano electrospray stage (custom-made, MTA for blueprints available by request from John Yates, Scripps Research Institute, La Jolla, CA; or Thermo Electron Nanospray II ion source; or Brechbuehler, Inc., Part # 1 2000 1000).
4. MicroTight Cross (UpChurch Scientific, Part # P-777; <http://www.upchurch.com/>).
5. Micro Ferrule for 360 µm o.d. tubing (UpChurch Scientific, Part # F-152).
6. Polyimide coated fused silica, 50 µm i.d. x 365 µm o.d. (Polymicro Technologies, <http://www.polymicro.com/>; Part # TSP 050375).
7. Gold wire 0.025" diameter (Scientific Instrument Services, <http://www.sisweb.com/>; Part # W352).

2.8.2. Solutions

1. Buffer A: acetonitrile/formic acid/double distilled H₂O, 5/0.1/95, v/v.
2. Buffer B: acetonitrile/formic acid/double distilled H₂O, 80/0.1/20, v/v.
3. Buffer C: 500 mM ammonium acetate in buffer A, filtered.

2.9. Data Analysis

2.9.1. Searching MS/MS dataset

SEQUEST™ (15) (Thermo Electron and John Yates, Scripps Research Institute, La Jolla, CA) and/or PEP_PROBE (19).

2.9.2. Assembling and comparing protein lists

DTASelect / CONTRAST (16) (available by request from John Yates, Scripps Research Institute, La Jolla, CA).

2.9.3. Appending transmembrane domain predictions

TMpred (20) (http://www.ch.embnet.org/software/TMPRED_form.html).

3. Methods

The first step in NE enrichment is the isolation of nuclei. Critical to this step and all subsequent steps is the fact that nuclei from different tissues have distinct densities; thus the concentration of sucrose in buffers may need to be altered or centrifugation steps lengthened if nuclear envelopes are to be isolated from tissues other than liver (*see Note 12*).

3.1. Preparation of Tissue (Rodent Livers)

Most of the nuclear envelope preparation procedure can be efficiently performed with one individual; however euthanizing and dissecting the animals should be done quickly and it is very helpful to have assistance at this point.

1. Overnight-fast the animals the night before procedure (*See Note 13*).
2. Euthanize rats or mice according to local animal protocols.
3. Immediately pull up on the ventral skin to isolate from the abdominal cavity. Make an incision anterior to posterior and two perpendicular incisions above the thoracic vertebrae and below the abdomen. Peel the skin back to access the liver.
4. Remove the liver with a scalpel being extremely careful to avoid the yellowish tube directly behind (*See Note 14*).
5. While clasped in the forceps rinse fresh livers quickly in the beaker on ice containing H₂O on ice and then immediately place in the pre-weighed beaker on ice containing 0.25 M SHKM buffer with freshly added PMSF.

6. Return to the laboratory with livers as soon as local animal protocols have been satisfied. If possible, have two people working at this point so that one can begin processing the material while the other deals with disposal and clean-up.
7. Weigh livers to determine the volume of buffer to be added for homogenization.

3.2. Preparation of Nuclear Envelopes

1. Pour off the buffer and resuspend the livers in fresh, ice-cold 0.25 M SHKM with freshly added protease inhibitors (*see Note 15*) at 2 mL of buffer for every gram of liver, e.g. 50 g of livers should be resuspended in 100 mL of buffer (*see Note 16*).
2. Use scissors to chop livers into small pieces in the beaker with buffer.
3. Pour into a 55-mL Potter-Elvehjem homogenizer and homogenize at 1,500 rpm in the cold, bringing the pestle to the bottom three times (*see Note 17*).
4. Rinse the homogenizer with buffer and add to the homogenate.
5. In a cold room, fold cheesecloth over four times and lay in funnel. Pour ~40 mL of crude homogenate through the cheesecloth (*see Note 18*).
6. As the flow slows, fold the cheesecloth over and roll a sterile pipette along the outside from top to bottom to squeeze the fluid out. A wash with buffer poured into the central cavity formed by the cheesecloth may increase yield slightly.
7. Remove to round-bottom centrifuge tubes (*see Note 19*) and underlay with a cushion of 30% SHKM using a 14 gauge needle and syringe. Pellet nuclei at 1,000 g in a swinging bucket rotor (e.g. 2,000 rpm in a Beckman Coulter J6MI floor model centrifuge) for 10 min at 4°C.
8. Remove the supernatant carefully as the pellets are very soft. Keep this supernatant if microsomal membranes are going to be prepared at the same time from the same tissue as they will be in this fraction.

9. Resuspend pellets in 2.3 M SHKM and homogenize in Potter-Elvehjem homogenizer with three more strokes of the pestle at 1,500 rpm. Rinse with the same buffer, accrue, and dilute with 0.25 M SHKM to a concentration of 1.9 M sucrose.
10. Aliquot by 25 mL into each SW28 ultracentrifuge tube and underlay with 5 mL of 2.3 M SHKM using a 14 gauge needle in a luer lock syringe (*see Note 20*).
11. Balance the tubes from top and spin in SW28 rotor for 60 min at 82,000 g (25,000 rpm).
12. Move to cold room and scrape the red layer at the top off with a spatula, then pour off the rest of the supernatant by rapid inversion. Keep the tubes upside down in the cold for 10 min to drain them. Then gently wipe out the inside walls of the tubes with a folded kimwipe (or equivalent towel), being very careful not to touch the pellet.
13. Insert a clean dry spatula without touching the walls of the tube and scrape out the nuclear pellet. It is important that the spatula be dry so that the pellet will cling to it. Remove, again avoiding touching the walls of the tube, and resuspend the pellet in 0.25 M SHKM with freshly added 2 mM DTT and protease inhibitors (*see Note 21*).
14. Resuspend the pellet using a loose (Wheaton B-type pestle, ~0.1- 0.15 mm clearance) dounce homogenizer until all aggregates are broken. Wash the homogenizer with the same buffer and remove to round-bottom centrifuge tubes. Underlay with 5 mL of 30% SHKM with freshly added 2 mM DTT and protease inhibitors as in step 6. Pellet nuclei by centrifugation at 1,000 g for 10 min. Decant by inversion as the pellet should be compact (*see Note 22*).
15. Resuspend in 0.25 M SHKM, dounce again, and take a small aliquot for counting nuclei in a hemacytometer and for step 17. Then repeat pelleting as in step 14.
16. During centrifugation count nuclei. The number of nuclei in the squares on the field should be multiplied by 10^4 (unless a different correction factor applies to your

hemocytometer) and by the total volume (in mL) in which cells were resuspended for centrifugation in step 15. This number should be divided by 3×10^6 , which is the number of nuclei in an OD. The formula is:

$$\text{OD}_{\text{total}} = [\# \text{ nuclei on grid} \times 10^4 (\text{nuclei/ ml}) \times \# \text{ ml}] / [3 \times 10^6 (\text{nuclei/ OD})]$$

17. Resuspend in 10% SHM with freshly added 2 mM DTT and protease inhibitors at 20 OD/ mL. Take an aliquot to compare nuclei to the aliquot saved in step 15. The cells should be observed to swell in the hypotonic SHM buffer.

18. Add 4 U/ mL DNase and 1 $\mu\text{g/ mL}$ RNase and incubate at room temperature for 20 min. Observe digestion on the microscope in parallel. The phase grey of the nuclei should diminish slightly (*see Note 23*).

19. Underlay the solution with 30% SHM with freshly added DTT and protease inhibitors. Spin 30 min at 6,000 g using a swinging bucket rotor (e.g. 5,000 rpm in a Beckman Coulter floor model J6MI centrifuge) (*see Note 24*).

20. Carefully aspirate off the supernatant (do not decant by pouring) as the pellet will be very soft (*see Note 25*).

21. Resuspend the pellet at 50 OD/ mL in 10% SHM. Add 20 U/ mL DNase and 5 $\mu\text{g/ mL}$ RNase and incubate at room temperature, carefully following the digestion in an aliquot under the microscope. When 90% of nuclei are no longer phase-grey, aliquot 4 mL of nuclear envelope solution (200 OD) each to centrifuge tubes (chosen for desired storage method) and spin at 6,000 g (5,000 rpm) for 30 min (no cushion).

22. Carefully aspirate the supernatants and immediately flash-freeze the pellets in liquid nitrogen and store at -80°C .

3.3. Preparation of Microsomal Membranes

1. The supernatant after pelleting of nuclei (step 7 in *Preparation of Nuclear Envelopes*) is supplemented with 0.5 mM EDTA to inhibit metalloproteases and subjected to a subsequent centrifugation at 10,000 g (11,500 rpm in a Ti45 Beckman Coulter ultracentrifuge rotor or 10,500 rpm in a JA25 Beckman Coulter centrifuge rotor) for 15 min to remove mitochondria (*see Note 26*).
2. The post-mitochondrial supernatant is mixed with 5 volumes (e.g. 1 mL + 5 mL) of 2.3 M SHKM containing 0.5 mM EDTA, 2 mM DTT, and protease inhibitors to achieve a final sucrose concentration of just under 2 M sucrose.
3. Float the microsomal membranes by pouring 35 mL of the diluted membranes in each Ti45 tube and overlay with 9.5 mL of 1.9 M SHKM with 0.5 mM EDTA, 2 mM DTT, and protease inhibitors and overlay this with 3 mL of 0.25 M SHKM with 0.5 mM EDTA, 2 mM DTT, and protease inhibitors. Centrifuge at 57,000 g (27,000 rpm) in Ti45 rotor for 5 h (*see Note 27*).
4. The microsomal membranes will be in the interphase between the 1.9 M sucrose phase and have a yellow-brown appearance (at least in liver). Above this band should be a clear phase on top of which will be a white flakey band. Below the 1.9 M to lower phase transition the media should begin taking on a pink shade. The yellow-brown microsomal membrane band can be recovered by extraction with a syringe either through tube puncture from the side with a needle and or by inserting the needle through the upper phase (*see Note 28*).
5. The membranes are diluted with 4 volumes of 0.25 M SHKM containing 0.5 mM EDTA, freshly added DTT and protease inhibitors and are pelleted at 152,000 g (44,000 rpm in a type 45 Ti, 48,000 rpm in a type 50 Ti, or 60,000 rpm in a TLA100.3 rotor) for 75 min (*see Note 29*).

3.4. Extraction of Fractions

1. Alkaline extraction. Resuspend 100 OD of nuclear envelope or microsomal membrane pellet on ice in 1 mL of 0.1 M NaOH. Immediately move to TLA100.3 ultracentrifuge tubes and pellet insoluble material at 104,000 g (35,000 rpm) for 35 min (*see Note 30*). Rinse the packed pellet rapidly with double distilled H₂O and either freeze at -80°C or directly process to digest for mass spectrometry.
2. Extraction with salt and detergent. Resuspend nuclear envelope pellet in salt/detergent buffer and incubate on ice for 15 min, move to TLA100.3 ultracentrifuge tubes and pellet insoluble material at 104,000 g (35,000 rpm) for 35 min. Rinse the packed pellet with double distilled H₂O and either freeze at -80°C or directly process to digest for mass spectrometry.

3.5. Determining the Purity/ Quality of Fractions

1. Western blotting, estimating a protein concentration of ~3 µg/ OD, can be used to track the partitioning of known nuclear envelope or microsomal membrane proteins during cell fractionation (Figure 1) and in subsequent extraction of nuclear envelopes. We generally use antibodies to nuclear lamins, the inner nuclear membrane proteins LAP1 and LAP2 (available from Babco), and the endoplasmic reticulum marker RIC6 (anti-ribophorin).
2. Electron microscopy can also be used to determine the quality of nuclear envelopes and microsomal membranes. For examples of clean preparations see (7).

3.6. Preparation and Digestion of Proteins for MudPIT

Salt and detergent extracted NE proteins from mouse and rat livers (*ca.* 225 μ g and 375 μ g proteins, respectively) are digested with endoproteinase Lys-C and Trypsin, while membrane pellets from NaOH-extracted mouse NEs and MMs are first solubilized in 90% formic acid and cyanogen bromide, before being further digested with endoproteinase Lys-C and trypsin.

3.6.1. Salt and detergent extracted NEs

1. Resuspend salt and detergent extracted NE pellets from mouse and rat livers in 200 μ L of 0.1 M ammonium bicarbonate, pH 8.5, 8 M urea.
2. To reduce disulfide bonds, add 1 M TCEP to 5 mM final concentration and incubate at room temperature for 30 min.
3. To carboxyamidomethylate free cysteines, add 500 mM IAM to 10 mM final concentration and incubate at room temperature for 30 min in the dark.
4. Add Endoproteinase Lys-C (at 1 μ g/ μ L) to a final substrate to enzyme ratio of 100:1 (w/w) and incubate at 37°C overnight with shaking.
5. Dilute sample to 2 M urea with 100 mM ammonium bicarbonate, pH 8.5.
6. Add 1 M CaCl₂ to a final concentration of 2 mM.
7. Add 10 to 15 μ L of trypsin beads at 37°C (approximate substrate to enzyme ratio of 100:1) and incubate overnight with shaking.
8. Spin samples at 15,000 rpm for 30 min.
9. Pull off supernatants (discard bead pellets).

3.6.2. NaOH extracted NEs and MMs

1. Under a fume hood, resuspend the membrane pellets in 100 μ L of 90% formic acid, 500 mg/mL CNBr; mix well by pipetting and leave overnight in the dark.
2. Add concentrated NH₄OH drop by drop on ice. Check pH using pH indicator strips after every 100 μ L of added NH₄OH. The final pH should be around 8.5, and the final

volume should be around 500 μ L, corresponding to a 3- to 5-fold dilution (*see Note 31*).

3. Add solid urea to 8 M.

4. The next steps are as in 3.6.1. 2 through 9, with the exception that IAM is added to 20 mM final concentration.

3.7. Packing and Loading of Microcapillary Column

After digestion, the peptide mixtures are usually in large final volumes. In particular, the CNBr/trypsin digestion, with its multiple dilution steps, can reach volumes of well over 1 mL, which would take a while to load onto traditional 100- μ m columns. To concentrate and desalt the samples, we use an off-line solid phase extraction on these peptide digests, before loading them onto custom-made microcapillary columns.

3.7.1. Solid Phase Extraction of Peptide Mixtures

1. Wash a SPEC-PLUS PTC18 cartridge with 50 μ L of MeOH (push the solution through using a 1-mL syringe).

2. Wash the cartridge twice with 400 μ L of 0.5% (v/v) acetic acid in water (after this step, do not allow the cartridge to dry).

3. Load the peptide mixture.

4. Wash the loaded cartridge four times with 400 μ L of 0.5% acetic acid.

5. Elute peptides off with 50 μ L of acetonitrile/acetic acid/water, 90/0.5/9.5, v/v.

6. Speed-vac the samples down to dryness.

7. Add acetonitrile/acetic acid/water, 5/0.5/94.5, v/v to a final volume of 20 μ L.

8. Store at -80°C or load directly onto a 100 μ m microcapillary column.

3.7.2. Double-Phase Fused-Silica 100 μ m Microcapillary Column

1. Place about 40 cm of 100 μ m i.d. x 365 μ m o.d. fused silica into P-2000 laser puller

and use heating/pulling cycle settings (*see Note 32*) such as to pull the capillary to about a 5 μm opening.

2. Make slurries of 5 μm Polaris C₁₈ Reverse Phase and of 5 μm strong cation exchange SCX material, both at about 15 mg/mL in 500 μL of methanol (*see Note 33*).

3. Pack fused silica column with 9-10 cm of 5 μm Polaris C₁₈ RP using the high pressure loading device (Figure 2A). Mark resin level in column with a marker (*see Note 34*).

4. Pack with 4-5 cm of 5 μm strong cation exchange material (Figure 2B).

5. Wash with methanol for at least 10 min.

6. Equilibrate in Buffer A for at least 30 min.

3.7.3. Off-Line Loading and Desalting

1. Load the sample onto the microcapillary column by placing the sample-containing Eppendorf tube in the high pressure device (Figure 2C).

2. Wash with Buffer A for at least 30 min using the high pressure device.

3.8. Liquid Chromatography in-line with Tandem Mass Spectrometry

3.8.1. Multidimensional Liquid Chromatography

1. Install the loaded and washed two-phase microcapillary column on the nanoelectrospray stage. Connect the microcapillary column, quaternary HPLC pump, gold wire through which a 2.4 kV voltage is applied to the liquid phase, and overflow tubing using a MicroTight Cross (Figure 2D).

2. Keep the HPLC flow rate constant at 0.1 mL/min throughout the chromatography. However, to achieve a slower flow rate at the tip of the column of about 200-300 nL/min, split the flow using a waste line consisting of 50- μm fused silica capillary cut

to about 40 cm (i.e. back pressure of ~40 bar).

3. Run a 12-step chromatography run (24 hours) on samples with the gradient parameters described in Figure 3. The chromatography is set up through and controlled by the Xcalibur™ instrument software.

3.8.2. Tandem Mass Spectrometry

1. Using Xcalibur™, set up the collision energy at 35%.
2. Implement an acquisition scheme such that a cycle of one full MS scan (from 400 to 1600 m/z) followed by three MS/MS events on the top three most intense ions is repeated continuously throughout the chromatographic elution time.
3. To allow less intense ions to be analyzed, enable dynamic exclusion for 5 min.
4. Convert each RAW file (one per chromatographic step) into a DAT file using the XCalibur file converter function.

3.9. Data Analysis

3.9.1. Searching the MS/MS dataset

1. To obtain the coordinates of the MS/MS spectra to be analyzed, convert each DAT file into a MS2 file using *extract-ms* (21) (Figure 4).
2. To remove spectra of poor quality and tentatively assign a charge state to precursor ions, apply the *2to3* software to MS2 files (22) .
3. To match MS/MS spectra to peptides, use SEQUEST™ (15) or PEP_PROBE (19) (see **Note 35**) against a database containing human, mouse and rat protein sequences downloaded from NCBI (National Center for Biotechnology Information): this was 106,360 sequences on April 24th, 2003, and was complemented with 172 sequences from usual contaminants (human keratins, IgGs, proteolytic enzymes). Set the

sequest.params file such as i) the peptide mass tolerance is 3, ii) no enzyme specificity is required, iii) parent ions are calculated with average masses, while fragment ions are modelled with monoisotopic masses, and iv) cysteine residues are considered fully carboxyamidomethylated and searched as bearing a static modification of +57 Da.

3.9.2. Assembling and comparing protein lists

1. To assemble and parse the peptide information contained in the SEQUEST output files, run DTASelect on SQT files (Figure 4).
2. To compare the proteins detected in salt and detergent-extracted NE, NaOH-extracted NE and NaOH-extracted MM samples, create a CONTRAST table (Figure 4). The validity of peptide/spectrum matches is assessed primarily with the PEP_PROBE-defined confidence for a match to be non-random (at least 85%). In addition, spectra passing this filter have to match peptides that are at least 7-amino-acid-long, with a normalized difference in cross-correlation scores (DeltCn) of at least 0.08, and minimum cross-correlation scores (Xcorr) of 1.8 for singly-, 2.5 for doubly-, and 3.5 for triply-charged precursors. Proteins identified by single unique peptides are allowed (see **Note 36**), while contaminants, human keratins and proteins that are subsets of others are removed from the final list.
3. To create subset databases, in fasta format, containing only the proteins in the final list, use the “Database” utility of CONTRAST (add “Database” to the [Options] field of Contrast.params file).
4. To generate tab-delimited text files linking proteins to peptides, using the “--DB” option in the [Criteria Sets] field of Contrast.params file (Figure 4).

3.9.3. Appending transmembrane domain predictions

1. Predict the number of transmembrane segments using TMPred on a FASTA file containing the amino acid sequences for all detected proteins. Set a minimum score of 1000 in one orientation and 1900 in both (*see Note 37*).

2. Build a table linking proteins and number of predicted transmembrane domains.

3.9.4. *Generating a list of putative nuclear envelope transmembrane proteins.*

1. Consolidate protein lists and TM table into a relational database using MSAccess.
2. Query for hypothetical proteins with transmembrane domains detected in either of the NE preparations but not the MM samples (Figure 4) (*see Note 38*).

4. Notes

1. As with most protocols there is an optimal middle-ground with too little or too much starting material resulting in lower yields. In our hands, ten to twelve rats produce optimal yields without saturating the sucrose gradients in a Beckman-Coulter SW28 rotor.
2. This mouse strain was chosen to obtain more tissue per animal because it has much greater than average body weight (~35 g at 9 weeks). When choosing a heavier strain it is important to check that the mice do not have diabetes-related problems as this could bias results, some NE diseases having associated diabetes defects. The CB6F1/J strain is free of such defects.
3. It is recommended to pre-weigh the beaker containing the SHKM buffer as it simplifies measuring the weights of the livers.
4. This will also be needed in the *Preparation of Nuclear Envelopes* section, except that different and additional protease inhibitors should be added then. The PMSF is added here because additional proteases (mostly serine) may be released onto the liver tissue during dissection.

5. Muslin will also do if not chemically treated: make certain to ask supplier.
6. MgCl_2 concentration in the original procedure was 5 mM throughout; however if nuclear envelopes are being prepared for viewing by electron microscopy, dropping the concentration through most of the procedure to 0.1 mM will yield better structure. However, during DNase and RNase treatment it is important to increase the MgCl_2 concentration back to 5 mM.
7. The optimal protease inhibitors will vary according to the tissue being investigated; so it is important to investigate what proteases are present at high concentrations in the tissue of choice. The choice for liver focuses on inhibiting serine, trypsin, cysteine, and aspartic proteases present in this tissue.
8. If general protease cocktails are used, it is important to make certain that they do not contain EDTA as this is bad for nuclear envelope preparation.
9. The solution can be prepared by adding 230 mL of an 85% (w/v) sucrose stock to 12.5 mL of 1 M HEPES-KOH, pH 7.4, 6.25 mL of 1 M KCl, and 1 mL of 1 M MgCl_2 , and freshly added 2 mM DTT and protease inhibitors. Other concentrations of sucrose can then be obtained by mixing the 2.3 M SHKM with the 0.25 M SHKM.
10. The stock solution can be prepared by mixing 67 mL of 0.25 M SHKM to 33 mL of 2.3 M SHKM.
11. Bulk material is not available. The resin is extracted by cutting the HPLC column in half with a hacksaw, then washed with methanol, dried, and stored as a powder.
12. Chapters detailing modifications of the nuclear envelope protocol for human blood lymphocytes and rat muscle are being prepared for other volumes in the Methods in Molecular Biology series.

- 13.** This has been experimentally shown to increase yields by 30-50% with this procedure and reduces the RNA/ DNA ratio (6).
- 14.** This tube is particularly rich in proteases, so it is important to avoid cutting it or mixing it with the isolated liver.
- 15.** As many protease inhibitors are short-lived, it is important to add them fresh to buffers shortly before use throughout the procedure
- 16.** One can avoid the weighing step and estimate 5 g per liver.
- 17.** This requires a reasonable amount of physical strength and one must take care to keep the homogenizer straight with the direction of the pestle or the homogenizer can break. Also never stop the pestle rotation while it is inserted inside the homogenizer with liquid or this also can become stuck or break due to the vacuum produced during homogenization.
- 18.** There is an enormous amount of collagen in rat liver and this will clog the cheesecloth and reduce the yield if the cheesecloth is overloaded. Therefore the homogenate from no more than 3 rats should be filtered through one four times-folded cheesecloth. Mice have much less collagen than rats and therefore roughly twice the amount of material can be used for the same amount of cheesecloth.
- 19.** We prefer these because the pellet tends to distribute widely, but conical tubes could also be used.
- 20.** Due to the high viscosity of the 2.3 M sucrose solution it takes several minutes for each tube if an 18 gauge needle is used. In contrast, with the wide bore size of the 14 gauge, this same procedure can be performed in 30 seconds. It is important to use a luer lock syringe because the viscosity of the solution can produce high pressure on the connection.

- 21.** It is critical to avoid touching the walls because they are rich with proteases that were associated with the floated membranes.
- 22.** This and step 13 are done to wash away some of the high amount of contaminating collagen prior to extraction of nucleoplasmic contents. These washes are necessary to prevent saturation of sucrose cushions when chromatin is released, but are only necessary for liver preparations and other tissues that are particularly high in collagen.
- 23.** Do not be concerned if the nuclei do not become phase-lucent at this point as this first treatment might be viewed as generally loosening rather than fully digesting the chromatin and, moreover, the digestion will continue during centrifugation.
- 24.** It is very important to use a swinging bucket rotor when spinning through the sucrose cushion at this point in order to float any chromatin that is released away from the nuclear envelopes.
- 25.** The supernatant will appear cloudy, but this is mostly chromatin that has been ejected and should give a dark, worm-like appearance under the microscope quite distinct from nuclear envelopes.
- 26.** The pellet here may be larger than the nuclear pellet.
- 27.** The membranes can be floated using either a fixed angle rotor or using a swinging bucket rotor (e.g. SW28). Better separation can be achieved if a second SW28 rotor and ultracentrifuge is available when nuclear envelopes and microsomal membranes are being prepared simultaneously. In this case, 20 mL of the diluted membranes are overlaid with 6 mL of the 1.9 M SHKM and this is overlaid with 2.3 mL of the 0.25 M SHKM and centrifuged at 57,000 g (~21,000 rpm) for 4 h.
- 28.** Extracting the microsomal membranes by side puncture is only possible if SW28 rotor and tubes are used, as the type 45 Ti rotor tubes are thick walled.

- 29.** The type 45 Ti rotor tubes must be filled to close to the top or they can collapse. There should be an orange pellet in the corner of the tube. Decant supernatant and freeze.
- 30.** It is critical to get this spinning immediately as loss of membrane proteins was observed by Western and membrane vesicles looked very fragmented by electron microscopy even after just 10 min sitting on ice prior to centrifugation (Tinglu Guan, personal communication).
- 31.** Because adding the base to the acidic sample causes “bubbling”, it is best to transfer the sample to a larger 15-mL conical tube in ice before neutralizing it, and then transfer it back to the smaller tube when the appropriate pH is reached.
- 32.** A typical four-step parameter setup for pulling approx 3 to 5- μm tips from a 100 μm i.d. \times 365 μm o.d. fused silica capillary is: [Heat = 290, Velocity = 40, and Delay = 200], [Heat = 280, Velocity = 30, and Delay = 200], [Heat = 270, Velocity = 25, and Delay = 200], [Heat = 260, Velocity = 20, and Delay = 200], with all other values set to zero.
- 33.** This concentration roughly corresponds to an amount of resin powder covering the tip of a small spatula, about 2-3 mm^3 .
- 34.** Using a black background behind the capillary being packed helps seeing the reverse phase and SCX resin levels inside the column.
- 35.** PEP_PROBE is a modified version of SEQUEST, which can calculate the confidence for a match to be non-random based on an hypergeometric probability model. Alternatively, False Discovery Rates (FDR) can be estimated by concatenating to the sequence database to be searched, randomized versions of each protein sequence (keeping the same amino acid composition and length). The theory is that for each spectrum matching a “shuffled” peptide (true negative)

there should be a false positive in the “normal” peptide dataset (23). Xcorr and DeltaCn values are then set such as to obtain peptide FDRs of less than 1%.

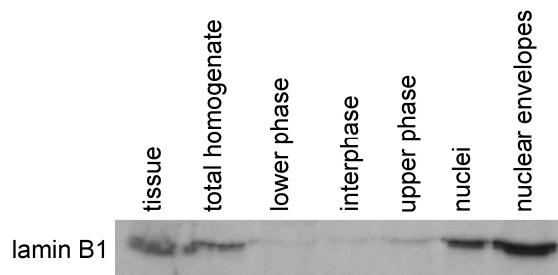
36. Although a two-peptides per protein cut-off allows for higher confidence, single peptide identifications are reported in the final dataset because some well-characterized NETs were detected by single peptides in previous studies, likely due to their lesser abundance.
37. These score limitations are based on an average to match those of the previously characterized NE proteins.
38. The selection criteria applied in this study (4) to discriminate true NET proteins from contaminating ER proteins is very conservative, since only proteins that were not found in MM fractions were considered for further analysis. However, differences in sequence coverage (percentage of a protein sequence covered by identified peptides) (24, 25) or spectral counts (number of spectra matching peptides from a protein) could be used as semi-quantitative parameters to determine whether a particular protein is “enriched” in a fraction of interest.

Acknowledgments

The authors would like to thank John R. Yates III in whose laboratory “shotgun” proteomics was developed, and Tinglu Guan in the laboratory of Larry Gerace from whom the nuclear envelope purification method was initially taught to Eric Schirmer. This work was supported by a Senior Research Fellowship to Eric Schirmer from the Wellcome Trust and by the Stowers Institute for Medical Research.

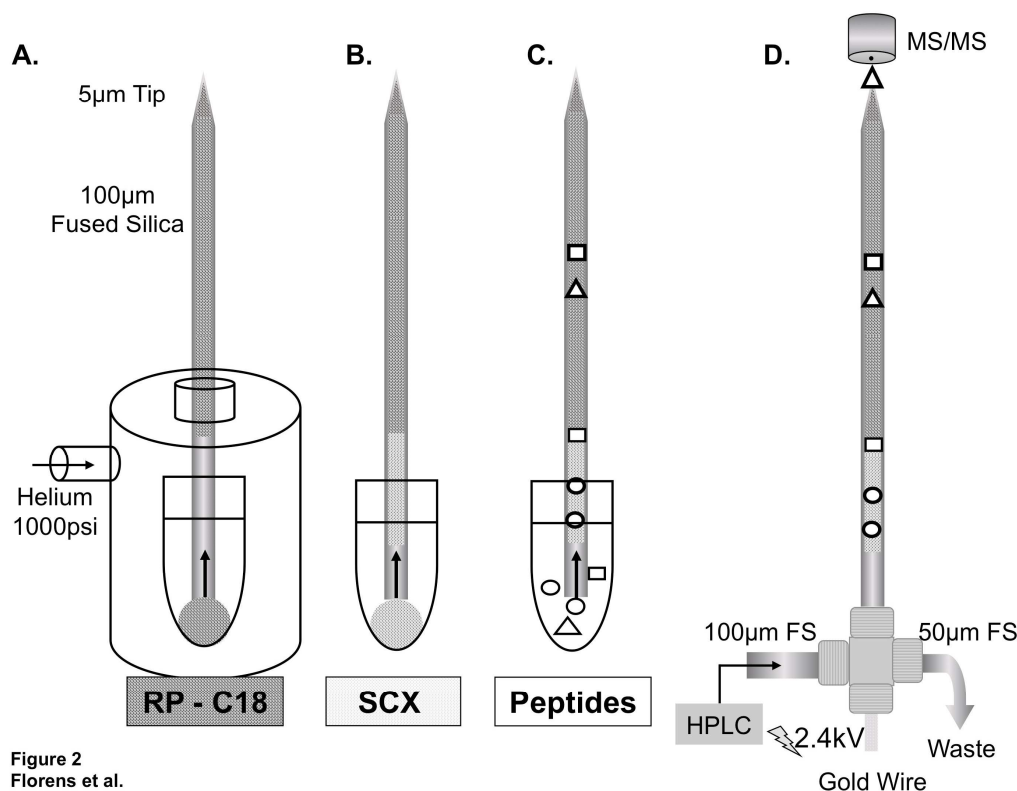
FIGURE LEGENDS

Figure 1.



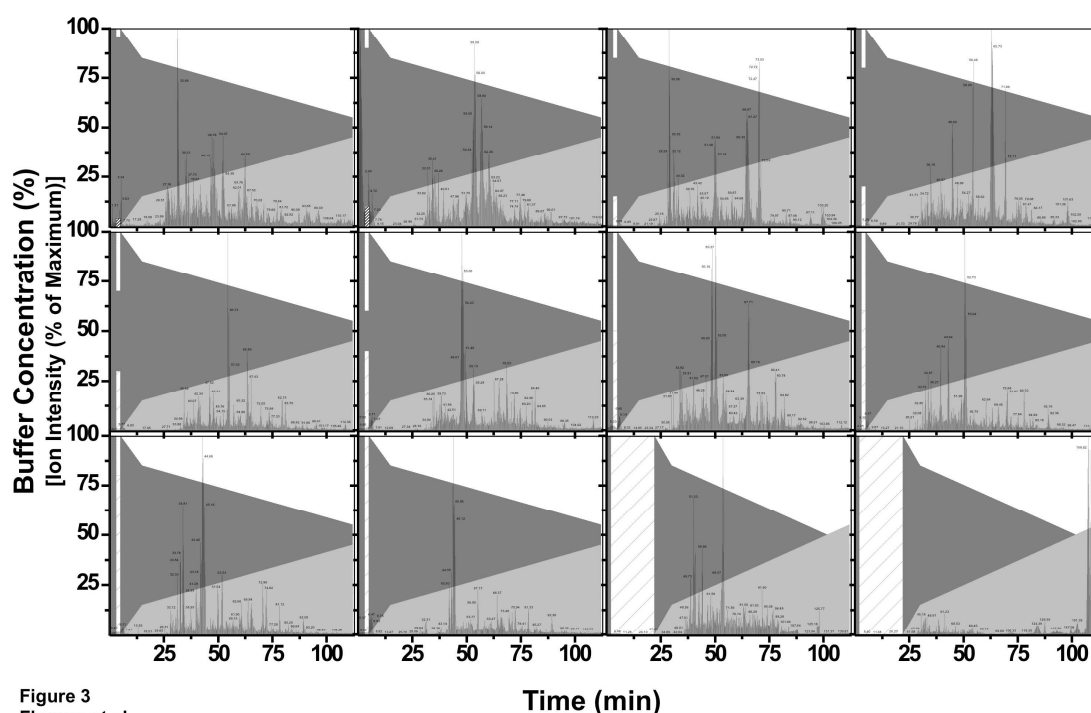
Enrichment of rat liver NEs during the procedure. Different fractions were kept during the NE purification and concentrated so that the same percentage of each fraction was loaded in gel sample wells. Fractions were resolved by SDS-PAGE, transferred to PVDF membranes, and reacted with antibodies to lamin B1, a well characterized NE protein. This NE marker was not notably lost during homogenization (compare “tissue” to “total homogenate”) and chromatin extraction steps (compare “nuclei” to “nuclear envelopes”). Moreover, lamin B1 was largely absent from the three phases through which nuclei pelleted in the sucrose gradient step (“upper phase”, “interphase”, and “lower phase”).

Figure 2.



Column Packing, Loading and Setup. A. A 100 μm fused silica capillary with a pulled tip is inserted into a high pressure device and packed using Helium pressure with a slurry of Aqua C-18 RP in methanol. B. The column is then packed with SCX material in a slurry, then washed with Methanol and Buffer A. C. The complex peptide mixture is pressure-loaded onto the column which is subsequently equilibrated in Buffer A. D. Loaded and washed column is installed in-line with a quaternary HPLC pump and a tandem mass spectrometer via a micro cross.

Figure 3.



Gradient Profiles for a 12-step MudPIT Run. Buffer A, B and C varying concentrations are represented by the dark gray areas, the light gray areas, and the striped white bars, respectively. A representative baseline ion chromatograph is shown in the forefront (black peaks) for each step (from the analysis of the complex peptide mixture obtained from mouse NaOH-extracted NEs). Each chromatographic step lasts 112 minutes. The salt concentration is equal to 4, 10, 15, 20, 30, 40, 50, 60, 80, and 100 % C in steps 1 through 10, respectively. In steps 1 through 10, the salt bump starts after 3min and lasts 2 minutes, while it lasts 20 minutes in the last two chromatographic steps. In steps 1 through 10, a rapid increase from 0 to 15% Buffer B occurs between 5 and 10 minutes, followed by a slow ramp to 45% B over 97 minutes. For steps 11 and 12, Buffer B concentration increases rapidly to 15% between 22 and 30 minutes, followed by a slow ramp to 55% B over 82 minutes.

Figure 4.

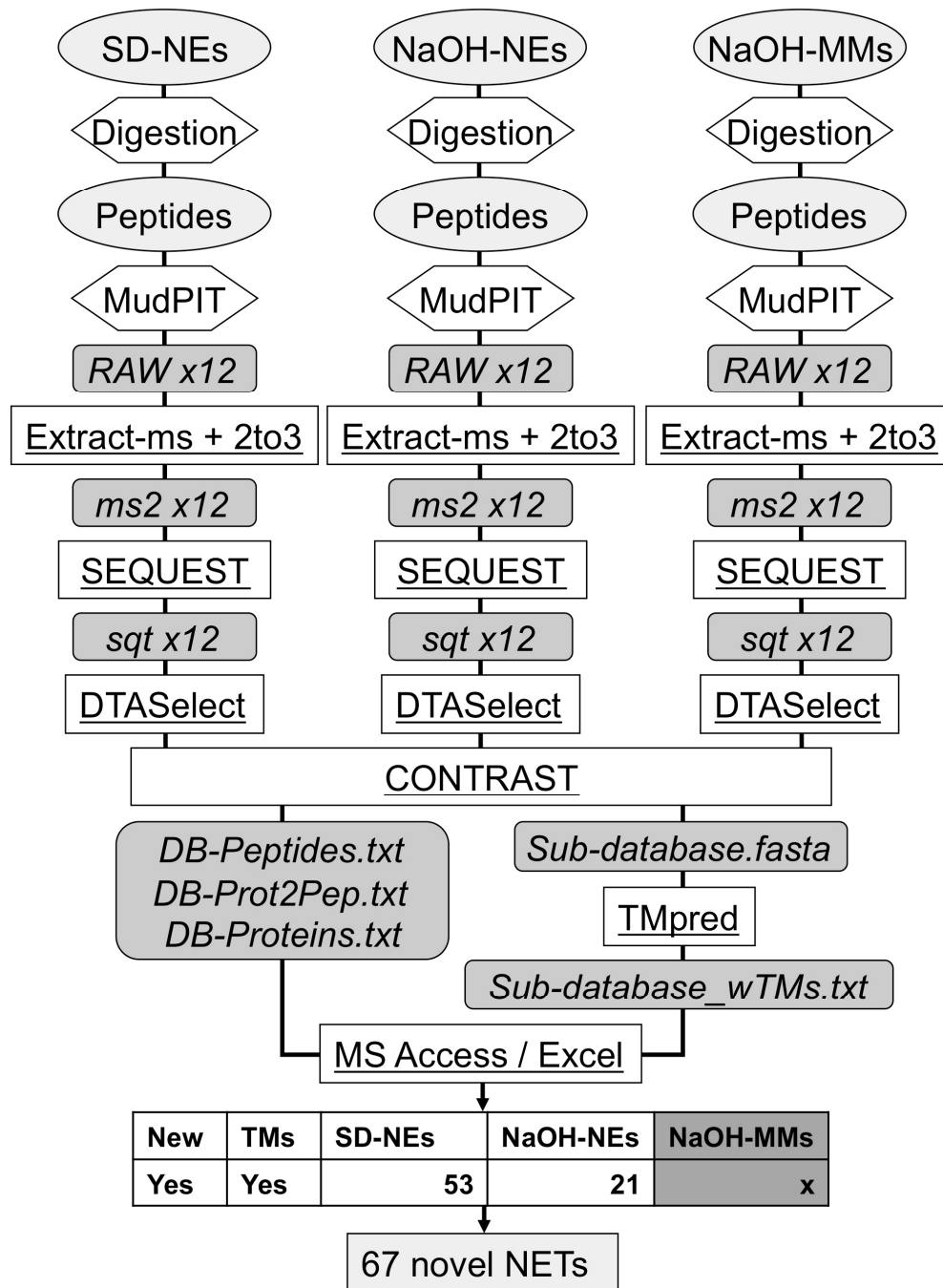


Figure 4
Florens et al.

Subtractive Proteomics Work Flow and Summary of Results. Protein and peptide mixtures are circled in light gray ovals, while processes are drawn within hexagons. Software are underlined in rectangles, while file types are italicized and in gray boxes. A total of 67 uncharacterized proteins with at least one predicted transmembrane

domain were found in at least one of the NEs preparations but not in the MMs runs. These are defined as putative nuclear envelope transmembrane proteins (NETs).

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